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The Conserved Third Transmembrane Segment of YidC Contacts Nascent *Escherichia coli* Inner Membrane Proteins^{*[S]}

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Escherichia coli YidC is a polytopic inner membrane protein that plays an essential and versatile role in the biogenesis of inner membrane proteins. YidC functions in Sec-dependent membrane insertion but acts also independently as a separate insertase for certain small membrane proteins. We have used a site-specific cross-linking approach to show that the conserved third transmembrane segment of YidC contacts the transmembrane domains of both nascent Sec-dependent and -independent substrates, indicating a generic recognition of insertion intermediates by YidC. Our data suggest that specific residues of the third YidC transmembrane segment α -helix is oriented toward the transmembrane domains of nascent inner membrane proteins that, in contrast, appear quite flexibly positioned at this stage in biogenesis.

Approximately 20% of the proteins encoded by the *Escherichia coli* genome are destined for the inner membrane. It is generally assumed that the Sec-translocon is required for the insertion of most inner membrane proteins (IMPs),² although a recent proteomic analysis showed that a defective Sec-translocon has surprisingly little effect on the steady state levels of most IMPs (1). The core of the Sec-translocon consists of the integral IMPs SecY, SecE, and SecG. SecD, SecF, and YajC form an accessory complex that facilitates protein insertion and translocation but is not essential for survival (2, 3). In addition, the essential IMP YidC was co-purified with the Sec-translocon (4). YidC has been characterized as an evolutionary conserved factor that is involved in the integration of many IMPs, including those that insert independent from the Sec-translocon (2, 3).

The exact function of YidC is unknown. Cross-linking studies have indicated that YidC contacts the transmembrane segments (TMs) of substrate IMPs upon their lateral exit from the Sec-translocon to facilitate their integration into the lipid bilayer (5–7). Recent studies on the biogenesis of CyoA (subunit II of the cytochrome *o* oxidase) have shown that YidC can

also function upstream of the Sec-translocon in the initial insertion of the N-terminal region of nascent CyoA followed by translocation of the (more complex) C-terminal domain by the Sec-translocon (8–10). Furthermore, YidC has been implicated in the folding and quality control of Sec-dependent IMPs (5, 7, 11, 12). Strikingly, YidC is also able to function as an insertase for small proteins such as F₀c (the subunit c of F₁F₀-ATPase) and M13 and Pf3 phage coat proteins (2, 3).

Little is known about the mechanism and timing of the recognition of substrate TMs by YidC both in its Sec-dependent and -independent operational mode. To address directly which region of YidC is involved in substrate recognition, we have used an *in vitro* site-specific cross-linking approach. We have found that during membrane integration, the TMs of several tested Sec-dependent and -independent IMPs are adjacent to a specific region in YidC TM3 that belongs to the most conserved part of YidC. The TM in the nascent substrate is flexibly oriented toward YidC, both lateral and vertical, whereas YidC TM3 displays a fixed orientation toward its substrate. We hypothesize that TM3 is (part of) a generic docking site for hydrophobic domains in growing nascent IMPs and provides a protected environment that facilitates their lipid partitioning and folding.

EXPERIMENTAL PROCEDURES

Enzymes, Reagents, and Sera—Restriction enzymes were from Roche Applied Science. Megashortscript T7 transcription kit was from Ambion Inc. [³⁵S]Methionine was from Amersham Biosciences. *Bis*-Maleimidoethane (BMOE) and *Bis*-maleimidoethane (BMH) were from Pierce. Benzophenone-4-maleimide (BPM) was from Molecular Probes. Phenanthroline, *N*-ethylmaleimide, and all other chemicals were supplied by Sigma. Antisera against YidC, PspA, and Lep have been described previously or were from our own collection (13).

Strains and Plasmids—Strain MC4100 was used to obtain translation lysate (14). Strain Top10F' (Invitrogen) was used for routine cloning and maintenance of plasmid constructs. Strain JS7131, in which *yidC* is under the control of the *araBAD* operator/promoter, was used to express YidC cysteine mutants (15). pCL1921YidC-StrepII-CBP derivatives encoding the YidC cysteine mutants were constructed as follows. The coding region for the calmodulin-binding peptide (CBP) was PCR-amplified from pCaKC (Stratagene) with a forward primer containing a SacI restriction site and the Streptavidin tag II sequence and with a reverse primer containing the EcoRI restriction site (primer sequences available upon request). The PCR product was digested with SacI and EcoRI and introduced 3' of the *yidC*

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

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² The abbreviations used are: IMP, inner membrane protein; BMOE, *Bis*-maleimidoethane; BMH, *Bis*-maleimidoethane; BPM, benzophenone-4-maleimide; CuPhe, Cu²⁺[phenanthroline]₂; IMV, inverted inner membrane vesicle; TM, transmembrane segment; CBP, calmodulin-binding peptide.

gene into pEH1YidC (16), resulting in pEH1YidC-StrepII-CBP. This plasmid was digested with KpnI and EcoRI, and the restriction product containing the 3' end of YidC gene with the StrepII-CBP tag was cloned into the low copy plasmid pCL1921YidC (17), replacing the 3' end of YidC, resulting in pCL1921YidC-StrepII-CBP (we will refer to this plasmid as pCLYidC). The plasmid pCLYidC was used as a template for constructing single cysteine mutants in the YidC gene using the QuikChange site-directed mutagenesis kit (Stratagene). First, the codon for cysteine 423 was changed into an alanine codon resulting in pCLYidC C423A. This plasmid was subsequently used as a template to construct further single cysteine mutants in *yidC* gene. The nucleotide sequences of the mutant genes were confirmed by DNA sequencing.

YidC wild type and cysteine derivatives (see Table 1) were expressed in the YidC depletion strain JS7131 carrying pEH3. pEH3 encodes the LacI repressor and is used to minimize leakage expression from the pCLYidC plasmid. Expression from pCLYidC was induced by 200 μ M isopropyl-1-thio- β -D-galactopyranoside. Strains were used to make IMVs for insertion assays (14). Single cysteine mutants of FtsQ, F₀c, and Lep were constructed in pC4Meth by nested PCR as described previously (18).

In Vitro Transcription, Translation, Integration, and Cross-linking—Truncated mRNA was prepared as described previously (4) from HindIII-linearized pC4Meth derivatives. Translation and membrane integration of nascent chains were carried out as described previously (4). Long range cysteine cross-linking was carried out with BMOE (1 mM) or BMH (0.5 mM) for 10 min at 25 °C and quenched with 5 mM β -mercaptoethanol for 10 min on ice. To separate integral membrane from soluble and peripheral cross-linked complexes, the samples were extracted with 0.18 M Na₂CO₃ (pH 11.5). The carbonate pellet fractions were analyzed by SDS-PAGE. Short range cysteine cross-linking was induced with 1 mM Cu²⁺[phenanthroline]₂ (CuPhe) for 10 min at 25 °C and subsequently quenched with 10 mM *N*-ethylmaleimide for 10 min on ice. CuPhe was prepared as described previously (19), and the carbonate-resistant fraction was dissolved in SDS sample buffer without dithiothreitol. For photocross-linking, nascent chains (prepared as described previously (4)) and BPM (1 mM) were incubated for 10 min at 25 °C and quenched with 10 mM *N*-ethylmaleimide on ice for 10 min. Subsequently, the nascent chains were purified through a high salt sucrose cushion (500 mM KOAc, 500 mM sucrose, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9) and resuspended in RN buffer (100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9). IMVs were added to allow targeting and integration into the membrane, and the samples were UV-irradiated to induce cross-linking to membrane components as described previously (4). The carbonate pellet fractions were analyzed by SDS-PAGE. Radiolabeled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473.

RESULTS

Experimental Strategy—To investigate the interaction of YidC with nascent IMPs during their insertion into the *E. coli* inner membrane, we have cross-linked integration intermedi-

ates in an *in vitro* approach (4). In this technique, translation of a truncated mRNA (a mRNA that lacks a stop codon) is translated in a cell-free *E. coli* extract to produce nascent, ribosome-bound polypeptides of predefined length. Translation is carried out in the presence of [³⁵S]methionine to label the nascent chains, and IMVs are added to allow co-translational insertion of the nascent IMP into the Sec/YidC-translocon or YidC insertase. Upon insertion, interactions of the nascent chain and the translocon/insertase are probed by cross-linking using homobifunctional cysteine specific reagents. Introduction of single cysteines in both the nascent chain and the interacting partner protein makes the procedure site-specific. Thus, this approach yields spatial information regarding the proximity of the integration intermediate and its partner at the residue level.

The integration intermediates used in this study were derived from the model IMPs FtsQ, leader peptidase (Lep), and the F₀c subunit of the F₁F₀-ATPase. FtsQ is a single spanning, type II IMP involved in cell division (see Fig. 3A) (20). Lep, the major signal peptidase, spans the membrane twice with translocated N and C termini (see Fig. 6A). F₀c has the same topology as Lep but comprises a much shorter translocated C terminus (Fig. 7A). FtsQ and Lep have been shown to use the Sec/YidC-translocon for membrane integration (4, 5, 15, 21). In contrast, F₀c only requires the YidC insertase (Fig. 7A) (22–24).

Construction and Characterization of Single Cysteine YidC Mutants—Using site-specific photo cross-linking, it has been shown previously that nascent FtsQ is targeted to the Sec-translocon by the SRP (18). The FtsQ TM inserts initially at SecY moving to a combined YidC/lipid environment upon elongation as suggested by sequential contacts during insertion of nascent FtsQ (6). For instance, a relatively long nascent FtsQ of 108 amino acids harboring a photo-probe at position 40 in the TM was shown to primarily cross-link YidC. Furthermore, 108FtsQ with a single cysteine engineered at position 40 was shown to cross-link YidC reconstituted in proteoliposomes together with SecYEG using the homo-bifunctional cysteine specific cross-linker BMOE (8 Å) (25). Because YidC only contains one cysteine at position 423 in the third TM, this observation implied that TM3 of YidC is close to the TM of nascent FtsQ at this stage in the integration process. Interestingly, TM3 belongs (together with TM2) to the most conserved regions in the Oxa1/Alb3/YidC family members (26).

To examine the proximity between YidC TM3/TM2 and nascent IMPs in more detail, we have used cysteine scanning cross-linking. First, the single endogenous cysteine in YidC was replaced by alanine to generate cysteine-free YidC. This derivative served as a negative control in our cross-link studies and as a starting point to introduce single cysteine residues at selected positions in TM3 and TM2 (Table 1), covering more than two turns of the presumably α -helical TM3 structure and one turn in TM2 (Fig. 1, B and C). All of the *yidC* constructs were cloned in the low copy expression vector pCL1921 under *lac* promoter control. To distinguish the plasmid-encoded YidC derivatives from endogenous YidC, a StrepII-CBP tag was added at the C terminus of all constructs, increasing the size of YidC by \sim 5 kDa. Tagged YidC that contains the cysteine at position 423 will be further referred to as wild type.

TABLE 1
Plasmids encoding YidC derivatives

Name	Plasmid	TM2	TM3
pCL	pCL		
YidC	pCL.YidC-StrepII-CBP		
C423A	pCL.YidC-StrepII-CBP C423A		C432A
F356C	pCL.YidC-StrepII-CBP C423A/F356C	F356C	C432A
S357C	pCL.YidC-StrepII-CBP C423A/S357C	S357C	C432A
I358C	pCL.YidC-StrepII-CBP C423A/I358C	I358C	C432A
F424C	pCL.YidC-StrepII-CBP C423A/F424C		C423A/F424C
P425C	pCL.YidC-StrepII-CBP C423A/P425C		C423A/P425C
L426C	pCL.YidC-StrepII-CBP C423A/L426C		C423A/L426C
Q429C	pCL.YidC-StrepII-CBP C423A/Q429C		C423A/Q429C
M430C	pCL.YidC-StrepII-CBP C423A/M430C		C423A/M430C
P431C	pCL.YidC-StrepII-CBP C423A/P431C		C423A/P431C

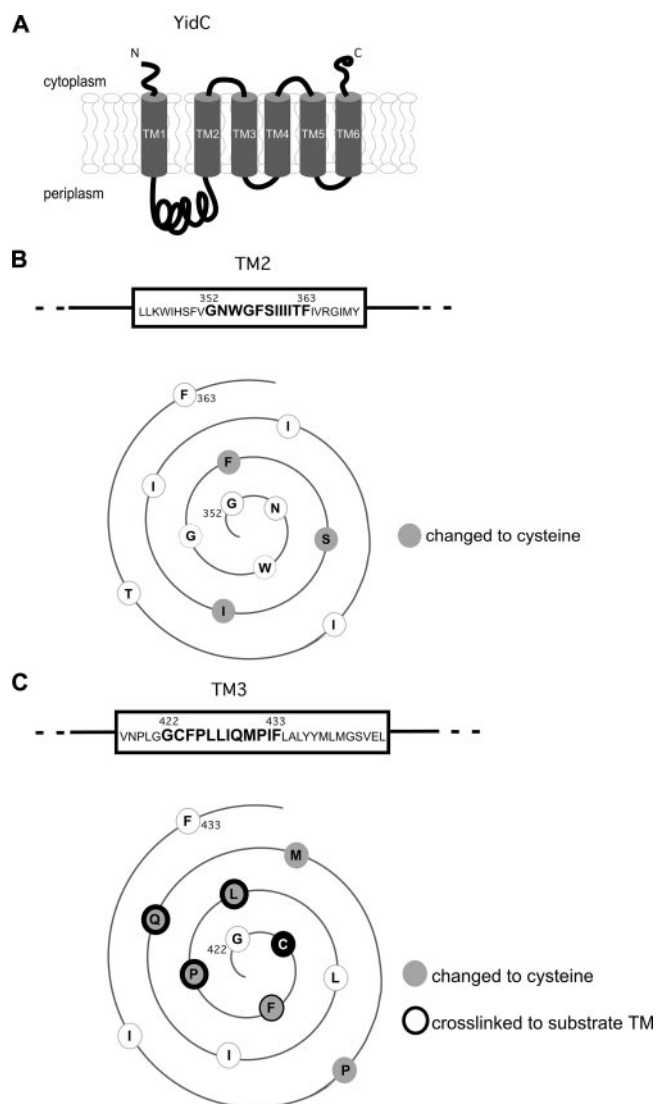


FIGURE 1. Schematic representation of YidC. A, topology model of YidC in the inner membrane. B, helical representation of YidC TM2 and TM3. C, high-lighted are the residues that have been changed to cysteine one at a time. The unique endogenous cysteine at position 423 (white on black) has been changed to alanine in the cases where another cysteine is introduced in YidC. The residues that cross-link to TMs in nascent chains are shown with a black ring. The thickness of the ring roughly corresponds to the efficiency of cross-linking.

Functionality of the YidC derivatives was assessed by investigating their ability to complement the *in vivo* growth defect that accompanies depletion of YidC. To this end, the constructs

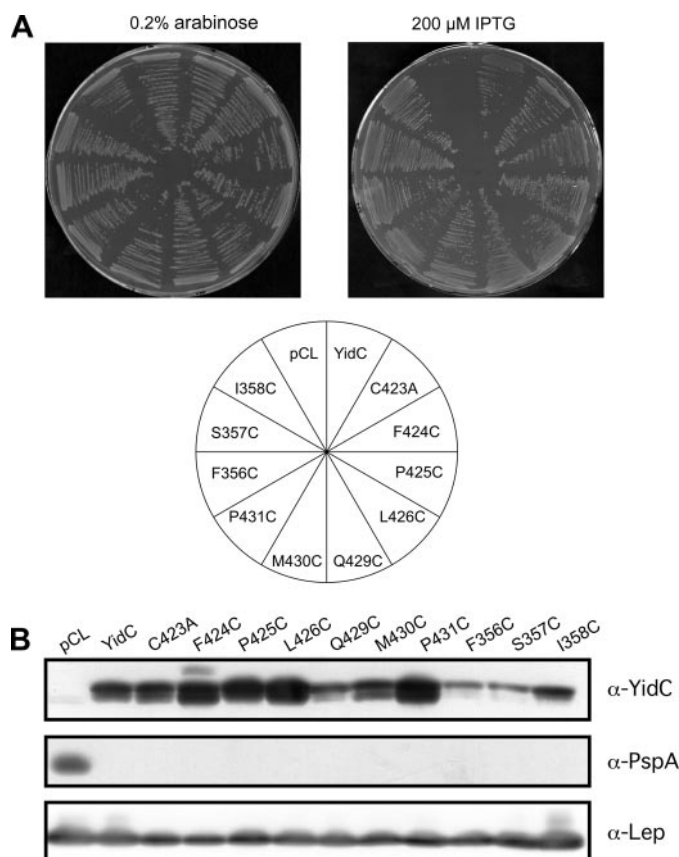


FIGURE 2. Functionality of YidC cysteine mutants. A, *E. coli* JS7131 containing pCLYidC cysteine mutants or empty vector were grown on LB agar plates. The cells were grown under conditions where the chromosomal *yidC* gene was induced (0.2% L-arabinose) or where the expression from the plasmid encoded YidC cysteine mutants was induced by isopropyl-1-thio-β-D-galactopyranoside (200 μM). B, Western blot analysis of IMVs containing the YidC cysteine mutants using antibodies against YidC, PspA, and Lep.

were introduced in the YidC depletion strain JS7131 in which expression of the chromosomal *yidC* is under control of the *araBAD* promoter (15). Complementation was assayed on solid medium in the absence of L-arabinose to repress the chromosomal *yidC* but in the presence of isopropyl-1-thio-β-D-galactopyranoside to induce expression of the plasmid-encoded *yidC* derivative (Fig. 2A). As a control, JS7131 transformed with the cloning vector only grew on the plate containing L-arabinose as expected. pCL1921 encoding wild type YidC complemented growth in the absence of L-arabinose, indicating that YidC with a C-terminal StrepII-CBP tag is functional, consistent with the permissive nature of the C-terminus of YidC with respect to alterations (16, 27). Importantly, all plasmid-encoded single cysteine YidC mutants were able to complement growth of JS7131 in the absence of L-arabinose, indicating that they are functional.

For *in vitro* cross-link experiments, IMVs were prepared of all JS7131 derivatives grown in liquid LB in the absence of L-arabinose and the presence of isopropyl-1-thio-β-D-galactopyranoside to specifically express the plasmid-encoded YidC. YidC was detected in the IMVs of all mutants, albeit at different levels for reasons that are unknown. As expected, the YidC mutants ran slower in SDS-PAGE compared with untagged YidC (faintly visible in the empty pCL vector control) (Fig. 2B).

YidC TM3 Binds Substrate

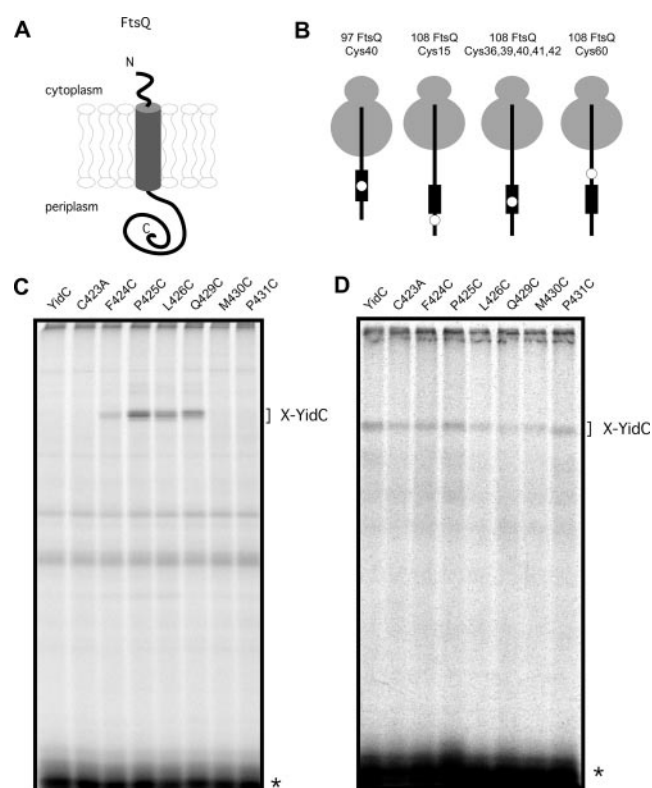


FIGURE 3. The TM of 108FtsQ inserts in proximity of YidC TM3. *A*, topology model of FtsQ in the inner membrane. *B*, schematic representation of the nascent FtsQ derivatives used in this study. The white dot indicates the position of the unique cysteine in nascent FtsQ, and the black rectangle represents the TM. *C*, nascent 108FtsQCys40 was synthesized *in vitro*, inserted in IMVs containing YidC with single cysteines in TM3, and cross-linked using BMOE or BPM. *D*, carbonate-resistant pellet fractions are shown. The nascent chains are indicated by asterisks, and the positions of YidC cross-linking adducts are indicated by brackets.

We have shown previously that depletion of YidC leads to up-regulation of PspA, which is in part associated with the inner membrane (13). PspA is a stress protein that responds to dissipation of the proton motive force (28). YidC depletion affects the proton motive force by defects in the functional assembly of the F_1F_0 -ATPase and the cytochrome *o* oxidase (13, 29). As shown in Fig. 2*B*, the induction of PspA seen in the empty pCL vector control is completely prevented by expression of all YidC derivatives. This indicates that all of the mutants are able to sustain the proton motive force and are functional in the assembly of respiratory chain complexes. Together, the data suggest that the YidC derivatives, although for unknown reasons expressed at different levels compared with the Lep loading control (Fig. 2*B*), are functional.

The TM of 108FtsQ Inserts in Proximity of YidC TM3—To probe the environment of the TM in the 108FtsQ integration intermediate near YidC TM3, 108FtsQCys40 (Fig. 3*B*) was inserted in IMVs that contain YidC derivatives with single cysteine substitutions in TM3 (Fig. 1*C*). Upon integration, the samples were cross-linked using the homo-bifunctional cysteine-specific reagent BMOE. Strong and specific cross-linking was observed to YidC (verified by immunoprecipitation, not shown) harboring a cysteine at positions 425, 426, 429, or, to a lesser extent, 424 (Fig. 3*C*). No adducts were observed in the absence of BMOE (not shown) or when using IMVs that con-

tain the cysteine-less YidC mutant (Fig. 3*C*), confirming the specificity of the procedure. Notably, the positions that strongly cross-link to 108FtsQCys40 seem to cluster at one side of the putative α -helical YidC TM3, although more positions should be analyzed to confirm this notion (Fig. 1*C*).

To rule out that, despite their functionality, some YidC mutants are structurally altered in such a way that they are no longer in proximity of nascent 108FtsQCys40, cross-linking was performed with the hetero-bifunctional reagent BPM. BPM reacts with the sulfhydryl groups of cysteine residues via a maleimide moiety. Upon ultraviolet irradiation, a reactive species is generated that can form covalent bonds with nearby groups. The linker arm connecting the two reactive groups is ~ 10 Å. 108FtsQCys40 nascent chains were produced, purified through a high salt sucrose cushion, and incubated with BPM to allow cross-linking to the Cys⁴⁰ residue. Subsequently, the samples were incubated with β -mercaptoethanol to quench cysteine cross-linking. Then IMVs were added for membrane integration of 108FtsQCys40-BPM, and the samples were UV-irradiated to induce cross-linking to membrane components independent of closely spaced cysteine residues. By this procedure, all of the YidC derivatives were cross-linked to the FtsQ integration intermediate (Fig. 3*D*). In this and the following experiments, no YidC cross-linking adducts were observed in the absence of cross-linkers (data not shown). This shows that the IMVs with the YidC derivatives are still functional in accommodating nascent 108FtsQCys40 in accordance with the *in vivo* complementation data described above.

The TM of 108FtsQ Is Dynamically Oriented Relative to YidC TM3—To obtain more spatial and structural information regarding the proximity of the TM in 108FtsQ and YidC TM3, four additional mutants were constructed that have single cysteines in the TM of 108FtsQ at positions 36, 39, 41, and 42 (Fig. 3*B*). These mutant nascent chains were integrated in the IMVs that contain YidC derivatives with single cysteine substitutions in TM3 (Fig. 1*C*) and cross-linked with BMOE. Interestingly, the cross-link patterns are very similar to that of 108FtsQCys40, *i.e.* cross-linking to YidC positions 425, 426, 429, and, to a lesser extent, 424 (Fig. 4*A*). Cross-linking is most efficient at position 425 except for 108FtsQCys36 where cross-linking peaks at position 426.

The similarity in cross-linking profiles for all of the tested positions in the TM of 108FtsQ indicates that the FtsQ TM does not occupy a fixed position relative to YidC TM3. Rather, the FtsQ TM seems to orient itself flexibly along the more rigid YidC TM3 perpendicular to the plane of the membrane. Importantly, the analyzed region of the FtsQ TM did not show a clear helical asymmetry in cross-linking to YidC TM3. This could mean that the TM in the FtsQ integration intermediate has not yet adopted an α -helical conformation but rather constitutes a randomly coiled stretch of residues that is able to reposition up and down relative to YidC TM3. Alternatively, the FtsQ TM is α -helical at this stage, but the helix has sufficient flexibility and space to rotate relative to YidC TM3. Finally, it cannot be excluded that the YidC TM3 docking site may have some mobility toward its substrate.

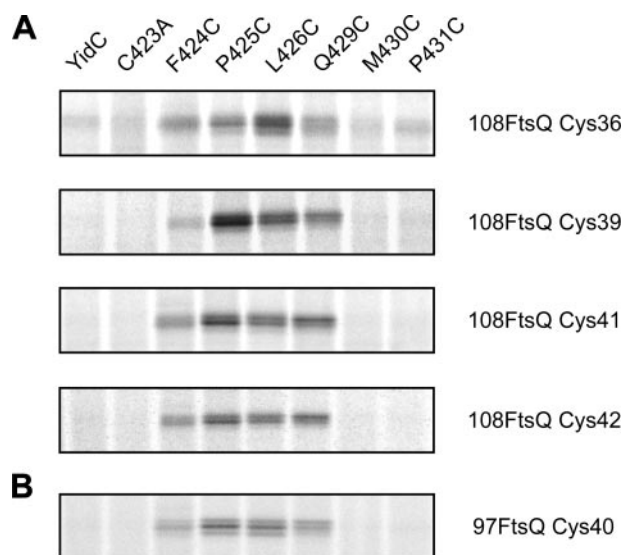


FIGURE 4. The TM of 108FtsQ is flexibly oriented toward YidC TM3 and held at in this position during protein elongation. *In vitro* translation of nascent FtsQ was carried out in the presence of IMVs containing YidC TM3 cysteine mutants and cross-linked using BMOE. Carbonate-resistant pellet fractions are shown. *A*, nascent 108FtsQ with a single cysteine in the FtsQ TM at positions 36, 39, 41, or 42. *B*, nascent 97FtsQ with a single cysteine at position 40 in the TM.

To verify that in this approach cross-linking to YidC TM3 is specific for the TM of 108FtsQ, single cysteines were introduced at positions 15 and 60 located in hydrophilic regions that flank the FtsQ TM. As expected, BMOE cross-linking yielded no YidC adducts (supplemental Fig. S1), consistent with previous photo cross-link data in which probes engineered at positions 10 and 59 of 108FtsQ did not show significant cross-linking to any region of YidC (4). Together, the data indicate that the TM of membrane-integrated 108FtsQ contacts YidC TM3 with a high degree of conformational freedom.

The TM of 108FtsQ Is Held at YidC TM3 during Membrane Integration—Next, we examined the effect of nascent chain length on the proximity between the FtsQ TM and YidC TM3. Previously, we have shown by photo cross-linking that the FtsQ TM in nascent chains as short as 97 residues contacts YidC (6). With our set of YidC cysteine mutants, we were now able to specifically examine the effect of nascent chain length on the interaction of the FtsQ TM with YidC TM3. Remarkably, 97FtsQCys40 integrated in the single cysteine YidC IMVs showed a BMOE cross-linking profile (Fig. 4*B*) almost identical to 108FtsQCys40 (Fig. 3*C*). These results suggest that the FtsQ TM, although flexibly oriented toward YidC TM3, is held at this position for at least some time during elongation and membrane integration. Although more lengths need to be analyzed to draw more definite conclusions, the data argue against a strictly linear insertion process in which the TM in the growing nascent chain slides through the Sec-translocon and YidC on its way into the lipid bilayer.

Close Proximity of Cysteine Residues in the TM in 108FtsQ to Specific Positions in YidC TM3—The previous experiments have shown that BMOE (spacer length of 8 Å) cross-links a cysteine introduced at position 40 in the TM of the 108FtsQ to cysteines placed at specific positions in YidC TM3. To examine the proximity between the cysteines in more detail, we used

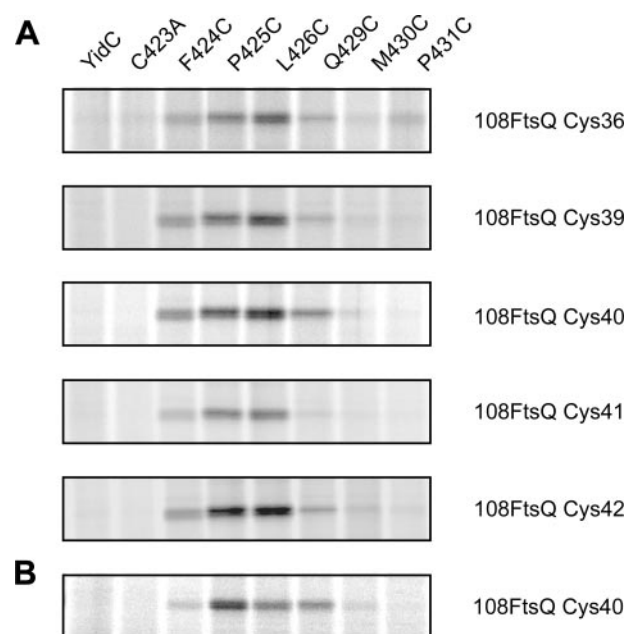


FIGURE 5. Close proximity of 108FtsQ TM to specific positions in YidC TM3. *In vitro* translation of nascent 108FtsQ was carried out in the presence of IMVs containing YidC TM3 cysteine mutants and were cross-linked using different cross-linkers. Carbonate-resistant pellet fractions are shown. *A*, nascent 108FtsQ with a single cysteine in the FtsQ TM at positions 36, 39, 40, 41, or 42 were cross-linked with the oxidizing agent CuPhe. *B*, nascent 108FtsQCys40 was cross-linked with BMH.

CuPhe. CuPhe forms a disulfide cross-link under oxidizing conditions between two cysteines provided that the β -carbons of the two residues are in close proximity (about 4 Å or less). Strikingly, the cross-link patterns obtained with CuPhe for 108FtsQCys40 (Fig. 5*A*) are very similar to those obtained with BMOE, *i.e.* cross-linking to YidC positions 425, 426, 429, and, to a lesser extent, 424 (Figs. 3*C* and 4*A*). This confirms the flexible and, at least temporarily, very close positioning of the TM in nascent 108FtsQ relative to specific residues in YidC TM3.

It is conceivable that the inability to cross-link 108FtsQCys40 to some positions in YidC TM3 is due to the short cross-linking span of BMOE. To examine this possibility, the homobifunctional cysteine cross-linker BMH (16.1 Å) was used. The results showed a similar cross-linking profile as with BMOE (Fig. 5*B*). No other tested positions in YidC TM3 came in reach of 108FtsQCys40. Taken together, these data suggest a rigid and confined substrate contact area in YidC TM3.

YidC TM3 Is Also Close to Lep (Sec-dependent) and F_0c (Sec-independent) Integration Intermediates—To investigate whether the proximity of the TM3 in YidC to the TM in FtsQ reflects a generic involvement of TM3 in substrate recognition and binding, we have analyzed the contacts of a Lep integration intermediate using cysteine cross-linking (Fig. 6*B*). Lep is a classical model IMP for Sec-dependent membrane insertion (30). Previous photo cross-linking studies have revealed that nascent Lep inserts at the Sec/YidC-translocon (5, 21, 31). In contrast to FtsQ (6), Lep is synthesized with a reverse signal anchor sequence (TM1) that is proximal to YidC at a very early stage in biogenesis approximately simultaneous with its earliest contact with SecY (5, 31). Nascent Lep of 50 or 72 residues with a photo-probe at position 10 in TM1 cross-linked efficiently and almost

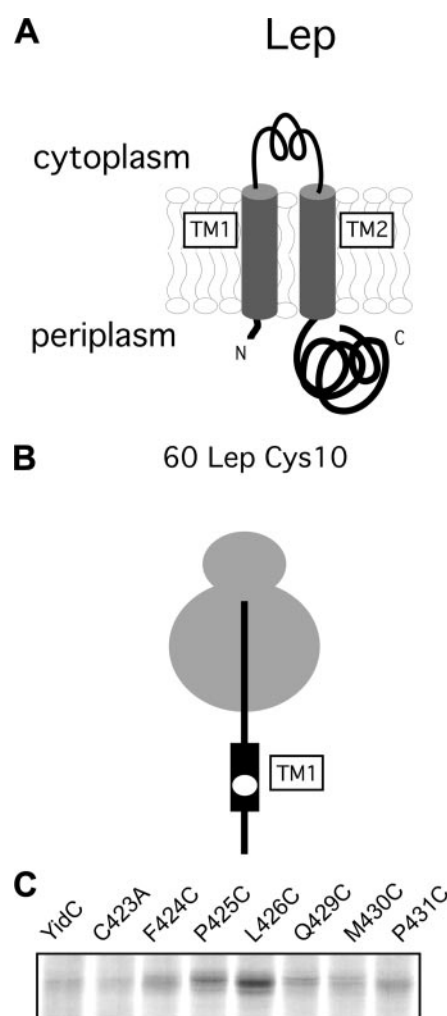


FIGURE 6. Lep cross-linking to YidC TM3 cysteine mutants. *A*, topology of Lep in the inner membrane. *B*, nascent Lep derivatives used in this study. The white circle represents the position of the unique cysteine at position 10 in nascent Lep. The black rectangle represents the TM. *C*, *in vitro* translation of nascent 60LepCys10 was carried out in the presence of IMVs containing YidC TM3 cysteine mutants and cross-linked using BMOE. Carbonate-resistant pellet fractions are shown.

exclusively to YidC (5). To study whether these contacts involve YidC TM3, a single cysteine was introduced at position 10 in a 60Lep construct and analyzed by BMOE cross-linking upon integration in the IMVs containing the single cysteine substitutions in TM3. Although less focused than the cross-link profile of FtsQ, cross-linking to YidC TM3 is evident and most efficient to positions 425 and 426, consistent with the FtsQ data (Fig. 6C).

To examine whether TM3 of YidC is also adjacent to the first TM of a Sec-independent integration intermediate, we have used F_0c as a model protein. F_0c , the c subunit of the F_1F_0 ATPase, is a small double spanning IMP that is thought to insert via the YidC-only pathway (22–24) (Fig. 7A). Consequently, nascent 79 F_0c , with a photo cross-link probe at position 15 in its first TM has been shown to almost exclusively cross-link YidC (24). Here, we introduced a single cysteine at this position and cross-linked nascent 79 F_0c Cys15 (Fig. 7B) upon integration in IMVs containing the single cysteine substitutions in YidC TM3. Again, significant cross-linking to resi-

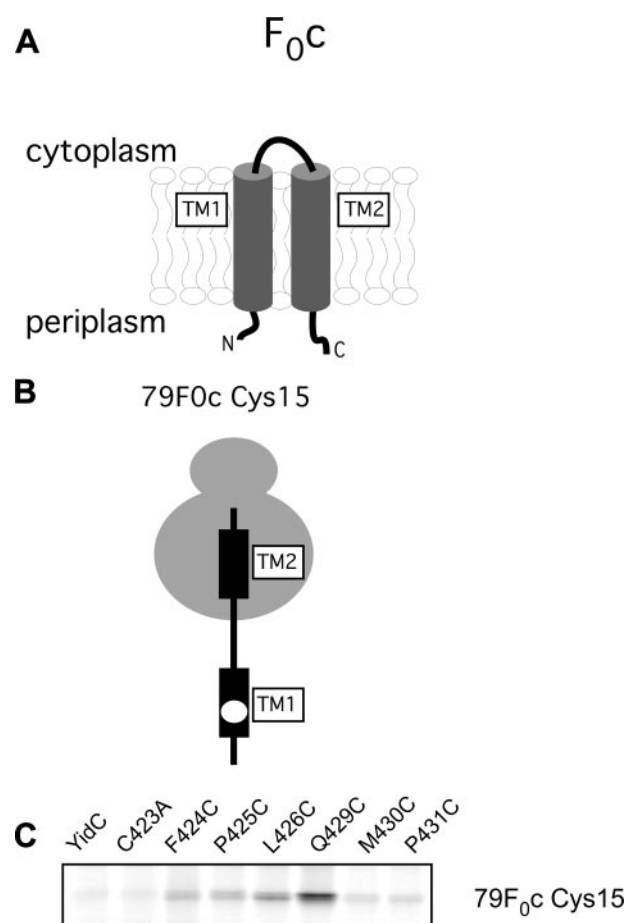


FIGURE 7. F_0c cross-linking to YidC TM3 cysteine mutants. *A*, topology of F_0c in the IM. *B*, nascent F_0c derivatives used in this study. The white circle represents the position of the unique cysteine at position 15 in nascent F_0c . The black rectangle represents the TM. *C*, *in vitro* translation of nascent 79 F_0c Cys15 was carried out in the presence of IMVs containing YidC TM3 cysteine mutants and cross-linked using BMOE. Carbonate-resistant pellet fractions are shown.

dues 424, 425, 426, and 429 was evident (Fig. 7C). However, in contrast to nascent FtsQ and Lep, the 79 F_0c Cys15 integration intermediate showed the most pronounced cross-linking to residue 429, indicative of a slightly shifted binding optimum. Together these data suggest that TM3 in YidC constitutes or participates in a “general docking site” for TMs in both Sec-dependent and Sec-independent nascent IMPs.

Cross-linking of YidC TM2 Cysteine Mutants—The previous data indicate that YidC TM3 is an important contact site for inserting nascent IMPs. Like TM3, TM2 is strongly conserved and cannot be replaced by an unrelated TM (27). To investigate whether TM2 also contributes to a docking site for TMs of nascent IMPs, single cysteines were introduced at positions 356, 357, and 358 that are expected to cover one helical turn near the center of TM2 (Fig. 1B). IMVs derived from cells expressing these YidC derivatives were subjected to BMOE cross-linking after integration of 108FtsQCys40, 60LepCys10, or 79 F_0c Cys15. 108FtsQCys40 did not show significant cross-linking to the TM2 positions tested (supplemental Fig. S2). Cross-linking with the Cys-UV cross-linker BMP yielded YidC adducts in all cases, confirming that YidC in these membranes is in contact with 108FtsQCys40 (results not shown). Similarly,

60LepCys10 and 79F₀Cys15 showed virtually no cross-linking to YidC TM2 with the exception of position 356, which is weakly cross-linked to 79F₀Cys15 (compare supplemental Fig. S2 with Figs. 6C and 7C, respectively). Although we cannot exclude the possibility that other regions in TM2 contribute to the contact with nascent integration intermediates, the combined data suggest that TM3 is the primary contact site for newly synthesized TMs.

DISCUSSION

In this paper we show that the conserved TM3 of YidC is adjacent to the first TM in IMPs during their integration into the membrane. Although more positions in both YidC and its substrates need to be analyzed, the data indicate that specific residues in the TM3 helix are rigidly oriented toward the substrate TM. In contrast, the TM of the nascent IMP appears to have some freedom of movement relative to YidC TM3. The TM of the IMP may occupy the contact site at YidC during synthesis until it is displaced into the lipid bilayer. A key element of our analysis is that the TMs in both Sec-dependent and Sec-independent substrates contact the same region in YidC TM3, suggesting a universal mechanism of substrate recognition by YidC.

YidC is an essential *E. coli* protein that has been cross-linked to nascent Sec-dependent and -independent IMPs, suggesting a generic role in the biogenesis of IMPs (2, 3). Functional studies demonstrated a key role for YidC in the insertion, folding, and quality control of IMPs (2, 3). Yet little structural information is available about YidC. The crystal structure of the major periplasmic domain in between TM1 and TM2 has been solved recently, but this domain is almost entirely dispensable for the insertase function (32, 33). Furthermore, a low resolution electron cryo-microscopy image of the native YidC protein has been presented but provides too little detail to offer mechanistic insight (34). In contrast, the structure of the Sec-translocon has been elucidated at relatively high resolution (3.2 Å), revealing a pore primarily embraced by two arms of SecY formed by TM1–5 and TM6–10 (35). It has been proposed, also based on biochemical evidence (36), that TMs leave the translocon laterally near TM2 and TM7 to become embedded in the lipid bilayer (35). It has also been proposed that accessory factors that assist in this process, such as YidC in bacteria and TRAM in eukaryotes, are located at this lateral gate to receive the TMs and transfer them in the right conformation into the lipids (2, 3, 37, 38).

Following this reasoning, our cross-link data using stalled integration intermediates indicate that YidC TM3 is oriented toward the lateral exit of the Sec-translocon, providing a hydrophobic interaction face that may facilitate the transfer of incoming TMs into the lipid bilayer. The “hot spot” for cross-linking is located at positions 425 and 426. Notably, this is a very conserved region in YidC and close to the G422R, C423R, and F424R mutations that confer a cold-sensitive phenotype (39). We cannot exclude the possibility that other regions in YidC contribute to substrate binding. Mutagenesis experiments have revealed that TM2, 3, and 6 are important for viability and functioning of YidC, although there is a remarkable tolerance toward single amino acid substitutions even at conserved resi-

dues (27). Interestingly, T362E in TM2 was isolated as a suppressor of C423R, pointing to an interaction between TM3 and TM2 (39). In our study, using cysteine cross-linking, we did not observe proximity of TM2 toward substrate TMs.

All of the tested cysteine substitutions in the TM of 108FtsQ show similar cross-linking to the single cysteine mutants of YidC TM3. This could indicate that at this stage in biogenesis, the substrate TM has not yet adopted an α -helical conformation. However, using fluorescence resonance energy transfer, it has been shown that the TM in a eukaryotic nascent membrane protein folds into a compact, presumably α -helical conformation already before reaching the Sec61 complex in the endoplasmic reticulum (40). It should also be noted in this context that previous photo cross-linking data (4) hinted at a periodic interaction between the FtsQ TM and YidC. However, because of the aspecific nature of the photoprobe, cross-linking to regions of YidC other than the target TM3 could not be excluded. Alternatively, the TM of FtsQ is α -helical at this stage but has some rotational freedom. A more speculative interpretation would be that YidC TM3 acts as a scaffold during membrane insertion of IMPs where TMs can fold into an α -helix prior to partitioning into the lipid bilayer. In any case, the efficient cross-linking of different FtsQ TM cysteine mutants with even short distance cross-linkers suggests a degree of lateral and vertical movement consistent with the fact that YidC interacts with a variety of substrate TMs with no apparent sequence similarity.

We have shown previously that the FtsQ TM interacts sequentially with SecY and YidC during membrane insertion (6). Interactions with YidC were already observed at 87–97 residues nascent chain length. Strikingly, the TM of 97FtsQ shows very similar cross-linking profiles as the TM of 108FtsQ, arguing that the TM is held for some time at YidC TM3 before being released into the lipids. Similarly, TM2 of nascent Lep has been shown to remain near YidC during elongation of 40–60 amino acids prior to release into the lipids (5). The extent and duration of the contact with YidC may depend on the characteristics and context of the individual TM in the substrate nascent chain. The first three TMs in the polytopic membrane proteins MtlA (7) and MalF (41) have been shown to contact YidC simultaneously, suggesting that YidC also functions as a platform for the assembly of complex IMPs. Possibly, YidC TM3 functions in the initial recognition of substrate TMs before relocating them for folding and assembly to other regions in YidC that remain to be determined.

YidC TM3 seems to act as a generic docking site for TMs in substrate nascent IMPs. The first TM in nascent Lep that, in contrast to the FtsQ TM, has a N-out orientation in the native protein, is also cross-linked to YidC TM3 during membrane insertion, albeit with a slightly shifted cross-linking optimum. More strikingly, YidC TM3 is cross-linked to the first TM of nascent F₀C that inserts independent from the Sec-translocon in the YidC insertase. This implies that the initial recognition of substrate TMs by YidC may be similar regardless of whether the substrate is approaching YidC from the Sec-translocon or directly from the cytosol. Although speculative, YidC might facilitate transfer of TMs from the interior of the Sec-translocon or the cytosol by a similar mechanism. Using photo cross-

linking, we have shown previously that the TM in nascent 108FtsQ shows simultaneous contacts with both YidC and lipids, suggesting that interaction may take place at a YidC-lipid interface (6). A comparable interface may be used by Sec-independent IMPs to facilitate insertion of their TM(s) into the membrane lipids concomitant with the translocation of small adjacent periplasmic domains. More detailed mechanistic insight will benefit from structural information on the conserved membrane-embedded domain of YidC purified in association with the Sec-translocon or as a separate entity preferably bound to a stalled nascent substrate IMP.

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